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Preliminary Method for Direct Quantification of Colistin Methanesulfonate by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

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Colistin use has increased in response to the advent of infections caused by multidrug-resistant organisms. It is administered parenterally as an inactive prodrug, colistin methanesulfonate (CMS). Various formulations of CMS and labeling conventions can lead to confusion about colistin dosing, and questions remain about the pharmacokinetics of CMS. Since CMS does not have strong UV absorbance, current methods employ a laborious process of chemical conversion to colistin followed by precolumn derivatization to detect formed colistin by high-performance liquid chromatography. Here, we report a method for direct quantification of colistin methanesulfonate by attenuated total reflectance Fourier transform infrared spectroscopy (ATR FTIR).

Colistin is an antibiotic of the polymyxin family. First available in the 1950s (1), its use diminished by the 1970s due to toxicity concerns and the advent of better-tolerated antibiotics. With the emergence of multidrug-resistant Gram-negative organisms, however, colistin has reappeared in clinical use (1, 2). Colistin is produced naturally by *Paenibacillus polymyxa* subsp. *colistinus* and consists of at least 30 separate components. For parenteral administration, it is converted chemically to a sulfonated prodrug, colistin methanesulfonate (CMS) (3). CMS is cleared renally, with a small fraction converted back to the active antibiotic *in vivo* in humans with healthy kidney function (4). CMS also converts readily to colistin in plasma *ex vivo* or in acidic aqueous solution (5, 6). Direct measurement of the prodrug in pharmaceutical formulations would reduce existing confusion in dosing regimens, (7, 8) and the ability to directly measure the prodrug and the active drug in plasma and tissues is important for better understanding of the metabolic characteristics of colistin. Like colistin, however, CMS is a heterogeneous mixture of molecules due to differing degrees of sulfomethylation, and this heterogeneity combined with weak UV absorbance has hindered the direct measurement of this increasingly used, last-resort antibiotic prodrug.

High-performance liquid chromatography (HPLC) with UV detection can be used to detect CMS but only at concentrations (0.5 to 5 mg/ml) above those expected in plasma after administration according to current dosing recommendations (9, 10). Recently, Nation and colleagues (5, 11–13) developed an HPLC-fluorescence method that enables detection at the physiologically relevant level (0.5 to 30 μg/ml) (4, 14, 15). In this process, CMS is converted to colistin, subsequently derivatized with fluorescent 9-fluorenylmethoxy carbonyl chloride (FMOC-Cl), and then quantified by HPLC with fluorescence detection. If the CMS is already partially converted to colistin, as in a degraded pharmaceutical preparation or a partially metabolized sample, the amount of colistin measured in the fully converted CMS is compared against the amount in the original CMS sample, which must also be derivatized using the same process (12). This method has become widely used and has enabled a number of studies that were not previously possible with HPLC methods (4, 16, 17). Liquid chromatography-mass spectrometry (LC-MS) methods that do not require fluorescent conjugation have also been developed (15, 18, 19). Like the HPLC-fluorescence detection method, these methods also measure CMS after it has been converted to colistin and measure only the two most abundant components as a proxy for the total amount of drug.

A rapid, simple, direct method of quantifying CMS in pharmaceuticals and in biological matrices may be useful in the standardization of CMS formulations, in pharmacokinetics studies, and potentially in therapeutic drug monitoring. Here, we demonstrate the direct detection and quantification of CMS in water and plasma using attenuated total reflectance Fourier transform infrared spectroscopy (ATR FTIR). This method measures chemical vibrations directly related to the CMS methanesulfonate group, allowing detection of all of the components of the prodrug, fully and partially methanesulfonated. FTIR has previously been used to detect trace drugs (20, 21), and the antibiotics cefixime (22) and trimethoprim-sulfamethoxazole (23) have been quantified using this technique. To our knowledge, quantitation of colistin or CMS by ATR FTIR has not previously been reported. This is also the first method that measures the prodrug CMS directly instead of as the active colistin antibiotic.

MATERIALS AND METHODS

Materials. Human plasma was purchased from Biological Specialty Materials (Colmar, PA). All water used in these studies was deionized water purified using an Aqua Solutions (Jasper, GA) Type I water purification system (resistivity, ≥18.2 MΩ). All other reagents were purchased from

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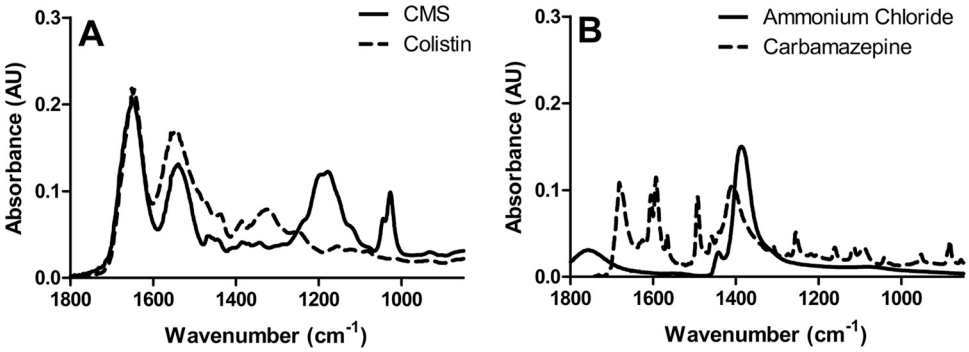

FIG 1 (A) FTIR spectra of CMS, with colistin shown for comparison. (B) FTIR spectra of the two internal standards, ammonium chloride and carbamazepine.

Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. The CMS used in this study was from Sigma-Aldrich (product C1511-10MU; lot BCBG8253V; ~11,500 U/mg).

Fourier transform infrared spectroscopy. All FTIR spectra were collected using a Thermo Scientific Nicolet iS10 spectrometer (Thermo Fisher Scientific, Madison, WI). The instrument was equipped with a Smart iTR attenuated total reflectance sampler containing a single-bounce diamond crystal. Data were collected and analyzed using OMNIC software (version 8.2). Spectra were collected in the 4,000 to 650 cm^{-1} range (0.5 cm^{-1} resolution and 32 scans per sample).

Preparation of samples for FTIR. To overcome low analyte signal intensity relative to water (a well-known problem in FTIR analysis [24, 25]), samples were dried on a solid substrate by evaporation. Aluminum foil circles were cut using a 2-mm biopsy punch and mounted on strips of laboratory film (Parafilm; VWR). For each sample type, 6 μl of sample was gently pipetted onto the aluminum and allowed to dry under ambient conditions. Once dry, the sample was inverted onto the ATR crystal, and the FTIR spectrum was collected.

CMS quantification from aqueous samples. CMS standards were prepared in water at 0.8 mg/ml and serially diluted to 0.003 mg/ml. Ammonium chloride (NH_4Cl, internal standard [IS]) was prepared at 0.25 mg/ml in water. These solutions were used to prepare FTIR samples containing between 4 ng and 4 μg of analyte and 0.25 μg of internal standard by combining 30 μl of each CMS standard with 6 μl of NH_4Cl internal standard and aliquoting 6 μl of this mixture onto each of five 2-mm aluminum foil circles. This calibration was repeated on three consecutive days.

CMS quantification in plasma. Several methods were examined to optimize the CMS extraction from plasma, including protein precipitation, reverse phase solid-phase extraction (SPE), weak anionic exchange SPE, and various combinations of protein precipitation with ultrafiltration techniques. The most reliable and effective method was the simple reverse-phase SPE method described below. Carbamazepine, which is retained on reverse-phase columns and therefore compatible with SPE, was used as the internal standard for this application instead of ammonium chloride, which is not retained on SPE.

CMS standards (500 μl) were prepared in plasma containing between 20 and 500 μg/ml CMS and 50 μg/ml carbamazepine (internal standard). Solid phase extraction (SPE) was performed on each sample according to the following protocol. SPE columns (Sep-Pak C_{18}; Waters, Milford, MA) were conditioned with 3 × 1 ml of methanol and equilibrated with 2 × 1 ml of water and 1 × 1 ml of 1 mM HCl. After equilibration, samples were loaded and columns were rinsed with 3 × 1 ml water and 2 × 1 ml 20% methanol in water. After briefly drying under a vacuum, samples were eluted into 1.5-ml microcentrifuge tubes using 2 × 250 μl methanol. Thirty microliters of 2:1 (vol/vol) methanol/water was added to each sample once dry. Samples were vortexed and sonicated briefly to solubilize dried material and centrifuged (1 min, 10,000 × *g*, 4°C) to precipitate remaining plasma proteins and collect the small sample volume at the bottom of the tube. Six microliters of each sample was added to one of five 2-mm aluminum foil circles. The peak area ratio of the five samples was averaged to give the calibration value.

CMS conversion samples. Twenty-five micrograms per milliliter of CMS containing 50 μg/ml carbamazepine (internal standard) was divided into 500-μl aliquots. Fifty microliters of 1 M sulfuric acid was added to each aliquot and neutralized with 80 μl of 1 M NaOH at 1, 2, 5, and 10 min. Solid phase extraction was as described above. Ten microliters of 2:1 (vol/vol) methanol/water was added to each sample once dry. Samples were vortexed, sonicated, and centrifuged as described above. Six microliters of each sample was added to an aluminum foil circle.

Colistin. Colistin sulfate was used to obtain an FTIR spectrum of colistin for comparison to CMS. Because the sulfate counterion absorbs strongly in the infrared, the molecule was desalted using a solid-phase extraction column to replicate the free base as would be found physiologically. An existing SPE method (11) was modified for this purpose. An SPE column (Sep-Pak C_{18}; Waters, Milford, MA) was conditioned with methanol and equilibrated with 1% sodium bicarbonate (pH 10). After loading, the sample was dried gently under a vacuum and washed with 3 × 1 ml water and eluted with 900 μl acetone. After drying, the sample was reconstituted in a 2:1 MeOH/water ratio and prepared using the aluminum foil method described above.

Preparation of samples for HPLC. For the aqueous calibration curve and the CMS conversion experiment, analogous HPLC experiments were performed using a previously described HPLC method (11, 12, 16). Briefly, CMS samples were converted to colistin using sulfuric acid, and the colistin was derivatized with FMOC-Cl during solid-phase extraction. Netilmicin was used as an internal standard. The HPLC apparatus (UltiMate 3000; Dionex, Sunnyvale, CA) consisted of a binary pump, sample injection loop, thermal-controlled column compartment (25°C), and UV-diode array with fluorescence detectors. The stationary phase was a reversed-phase Luna C_{18} column (150 × 4.6 mm, 100 Å, 5-μm particle size; Phenomenex, Torrance, CA), and the mobile phase consisted of a quaternary mixture (78:10:8:4 [acetonitrile/methanol/tetrahydrofuran/water]) with a flow rate of 1 ml/min. FMOC-colistin was detected by fluorescence using excitation and emission wavelengths of 260 and 315 nm, respectively. The results obtained were compared with those obtained by FTIR methods.

Statistical analysis. Each data point on the FTIR calibration curve was collected with $n = 5$ replicates on three consecutive days to determine interday and intraday variation. The limit of quantification of the assay was set at the lowest concentration at which the deviation from linearity was >20%.

RESULTS

FTIR spectra of colistin, CMS, and internal standards. Midrange FTIR spectra of CMS and colistin are shown in Fig. 1A. Charac-

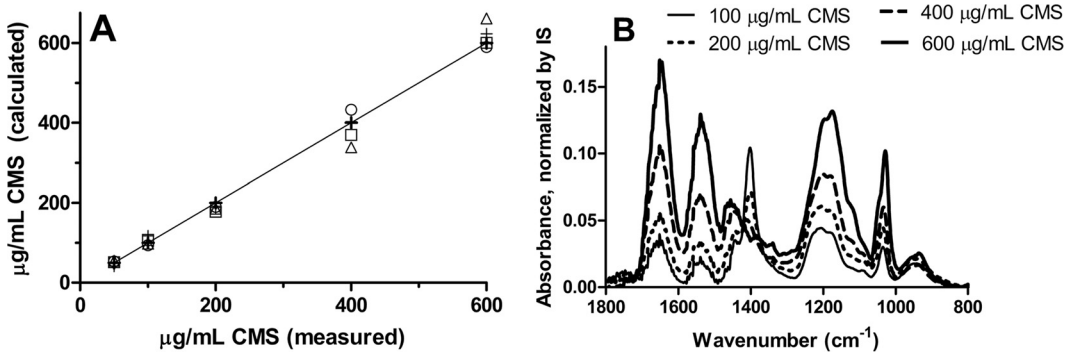

FIG 2 (A) Goodness of fit plot with three sets of FTIR calibration standards (open symbols). HPLC calibration standards (+) are shown for comparison. (B) Representative FTIR spectra normalized by the area of the internal standard peaks.

teristic FTIR absorbance peaks were identified for CMS, corresponding to vibrations produced by the methanesulfonate group and its linkage to the colistin (1,030 cm^{-1} S-O stretch and 1,150 cm^{-1} C-N stretch bands, respectively). Spectra between 2,000 and 4,000 cm^{-1} are dominated by broad hydrogen bonding bands not useful for peak analysis (data not shown). Spectra of the two internal standards are shown in Fig. 1B. Ammonium chloride has one strong sharp peak, the ammonium deformation between 1,425 and 1,275 cm^{-1}. Carbamazepine has several strong absorbance regions between 1,700 and 1,300 cm^{-1}, with weak but non-negligible peaks also apparent in the region between 1,300 and 1,000 cm^{-1} used for CMS measurement.

CMS quantification in water. Peak areas were integrated between 1,275 and 1,130 cm^{-1} and 1,045 cm^{-1} and 980 cm^{-1}, and the sum of these areas was used as the proxy for the CMS analyte. For the ammonium chloride internal standard, peak limits were set at the minimum or inflection nearest to 1,480 cm^{-1} and at 1,275 cm^{-1}. The center of the internal standard (IS) peak shifted between 1,390 cm^{-1} and 1,440 cm^{-1} depending on the amount of CMS in the sample, as shown in Fig. 2B.

The analyte/IS peak area ratio increased with the mass of CMS. A linear fit to the raw data did not provide satisfactory results ($R^2 < 0.90$ and structured residuals) at any analyte range. However, transforming the *x* values (micrograms analyte) using a natural log (ln) function linearized the relationship between the mass of CMS and the peak area ratio. Concentrations between 0.8 mg/ml (4 μg/sample) and 0.05 mg/ml (0.25 μg/sample) are shown in Fig. 2. The lower limit of quantification (LLOQ), 0.05 mg/ml or 0.25 μg/sample, was set at the lowest concentration where the deviation from this linearity was less than 20%. Table 1 gives the precision and accuracy parameters for this method.

TABLE 1 Accuracy and reproducibility of ATR FTIR method for aqueous solutions[a]

Calculated value (μg/ml)	Measured value (μg/ml)	% error	Variation (%)	
			Intraday ($n = 5$)	Interday ($n = 3$)
600	620	3	7	6
200	184	8	12	3
50	54	8	17	3

[a] Parameters are based on testing five replicates of each sample on each of three separate days.

Extraction and detection of CMS in human plasma. An unusually large volume of solvent (3 ml of methanol and 3 ml of aqueous solution) was required for the SPE conditioning and equilibration to remove silica-like artifacts in the final FTIR samples. Initial testing demonstrated that CMS and carbamazepine eluted in the 100% methanol fraction. A 50% methanol wash removed much of the CMS but not the more hydrophobic carbamazepine. In spite of carbamazepine's greater hydrophobicity and different charge profile, it performs adequately as an internal standard in this context.

After resuspension of the SPE product in 30 μl methanol-water, each sample was divided into five aliquots to gauge the precision of the assay. Surprisingly, there was a large amount of variability (coefficient of variation = 0.4 to 1) between these identical samples, but when the average values were calculated, a good linear fit was obtained between the average peak area ratio and the CMS concentration, as shown in Fig. 3. Subtraction of the internal standard spectrum was required due to peak overlap at lower analyte concentrations. However, the spectra in Fig. 3 show that these subtracted spectra have characteristic CMS peaks. SPE-processed samples were assayed by HPLC, showing no detectable colistin signal. Therefore, CMS conversion to colistin during this process was not significant.

Detection of CMS-to-colistin conversion by FTIR. The analyte/IS peak ratio dropped sharply in the first 5 min of acid exposure, indicating rapid loss of the methanesulfonate from the CMS molecules as it is converted to colistin. After 10 min, no detectable CMS remained. Figure 4 shows the time course of the conversion. HPLC performed in parallel using FMOC derivatization and a netilmicin internal standard, as described by Li et al. (12), showed a concomitant increase in formed colistin in similar samples. The internal standard (carbamazepine) was also tested alone to verify that its FTIR spectrum was not altered after exposure to acid.

DISCUSSION

CMS preparations for clinical use are labeled in either international units (IU) or colistin base activity (CBA), neither of which directly measures the actual quantity of CMS delivered (2). IU and CBA reflect microbiological activity, but CMS itself is not microbiologically active (26). These subtleties contribute to confusion regarding appropriate dosing, as recently reviewed (7, 8). Also, in spite of significant recent progress in our understanding of colistin (27), there remains considerable interest in the relationship be-

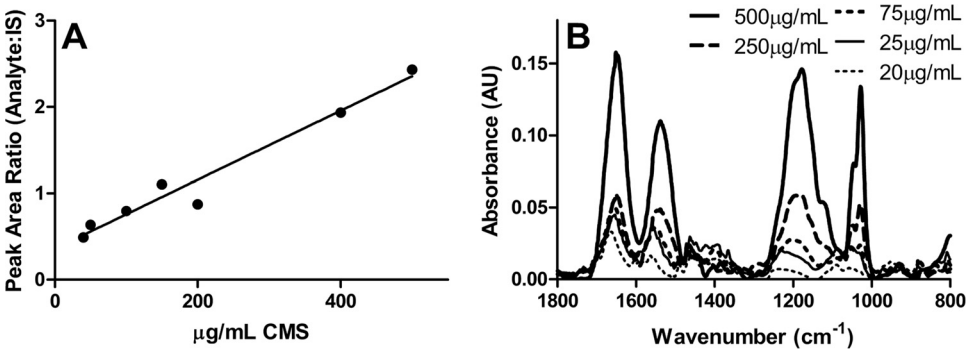

FIG 3 (A) Calibration curve showing linear fit ($R^2 = 0.96$) using SPE and FTIR. (B) CMS spectra after subtraction of carbamazepine internal standard. The CMS peaks are clearly visible.

tween CMS dosing, concentration in the bloodstream, formed colistin levels, and clinical outcome (4, 28, 29). The FTIR method described here is aimed at providing an assay capable of rapid, simple, and accurate quantification of CMS in water or plasma. Developed further, this assay may be used to compare levels of CMS methanesulfonation in pharmaceutical formulations, fill in pharmacokinetic knowledge gaps, and potentially serve as a platform for therapeutic monitoring of this important last-line antibiotic. To our knowledge, this is the first report of an assay that directly quantifies total CMS without requiring prior conversion to colistin.

The data in Fig. 1 and 2 demonstrate that it is possible to identify and quantify CMS with ATR FTIR, with sensitivity down to 50 μg/ml. Using the method described in this paper for measuring aqueous solutions, in which 5 μl of aqueous sample are combined with 1 μl of ammonium chloride internal standard, aqueous concentrations can be directly measured with a very small amount of sample. Preconcentrating aqueous samples using lyophilization may allow accurate measurement of even more dilute solutions, e.g., a 0.5 μg/ml solution can be measured by concentrating 1 ml of solution to 10 μl. For comparison, Coly-Mycin M, a pharmaceutical preparation used in the United States, is reconstituted in sterile water at a concentration of 75 mg/ml colistin base activity (CBA) (30), which is equivalent to approximately 200 mg/ml of CMS (7). In its current form, the assay is sufficiently sensitive to directly measure CMS concentrations in reconstituted drug formulations.

A method to directly measure CMS levels in the bloodstream without interference from formed colistin would enhance data gathered in human pharmacokinetic studies. Figure 3 demonstrates the quantification of CMS in plasma using ATR FTIR. The assay can currently detect CMS at 20 μg/ml in plasma, while CMS levels in human pharmacokinetic studies have been reported in the range of 0.5 to 30 μg/ml (4, 14, 15). Since samples are concentrated in the SPE process, the sensitivity can be increased by using a larger volume of plasma for analysis, but the assay in its current form is not sufficiently sensitive for use in clinical pharmacokinetics. The subtraction of the carbamazepine spectrum during analysis also introduces small artifacts that reduce performance at these lower concentrations, suggesting that the use of an alternate internal standard with less absorbance in the analyte region might help. The use of partial least-squares analysis instead of the analyte/internal standard peak ratio may also increase the sensitivity of the assay (22, 23, 31), allowing quantification across the entire physiological range of concentrations.

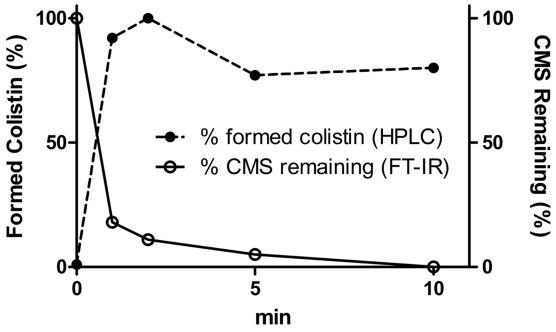

FIG 4 Conversion of CMS to colistin in 0.1 M sulfuric acid (aqueous solution). Loss of the methanesulfonate groups is rapid and parallels the appearance of formed colistin using HPLC.

ATR FTIR is generally regarded as a rapid analysis technique and, thus, has some advantages over HPLC (31, 32). Compared to the 15- to 30-min run time required for HPLC analysis of colistin (5, 11, 12), the collection time for each FTIR spectrum in this study was approximately 1 min. No mobile phase or analytical column is required for the technique, leading to potential cost savings. Sample preparation time, including the SPE required to separate CMS from plasma, was comparable to that required for HPLC. Ultraperformance liquid chromatography mass spectrometry (UPLC MS) run times can be much shorter, as little as 2 min, with less sample preparation time required (33); however, this equipment is more expensive than either FTIR or HPLC with fluorescence detection. The efficiency of this ATR FTIR method is limited by the high intraday variability (shown in Fig. 1). Because of this variability, five replicates at each concentration were used to generate the data in Fig. 2 and 3. This is likely due to uneven drying of the sample on the aluminum substrate and may be mitigated by surface modification, reducing the time of analysis even further.

The time course experiment illustrated in Fig. 4 demonstrates the difference between what is detected by published HPLC methods (the formation of colistin from CMS) and what is detected by the FTIR method described here (intact CMS molecules and the methanesulfonate itself). It also shows that the two methods produce compatible results. As shown in Fig. 4, the amount of CMS as measured by FTIR decays exponentially over time, as expected for this type of reaction. The HPLC-derived curve mirrors the FTIR

curve in the first 2 min of the reaction, during which colistin is generated exponentially as the CMS is converted to colistin. Notably, the amount of colistin detected actually decreases at later time points, possibly due to degradation of the colistin A or B by further acid exposure (34, 35). This experiment demonstrates the utility of the FTIR assay in following the conversion of CMS to active colistin and illustrates how FTIR can complement HPLC to develop a more complete view of CMS and colistin pharmacokinetics.

There are several limitations to the technique as currently developed. First, the distribution of methanesulfonate groups on the Dab residues of colistin cannot be evaluated, and unlike in HPLC, the ratio of colistin A and colistin B cannot be estimated. However, used in tandem with conversion to colistin and subsequent HPLC, this technique may be used to estimate the total degree of methanesulfonation, information that can help in standardizing pharmaceutical formulations of this prodrug. Furthermore, this technique may be used to investigate variation between manufacturers and batches and coupled with HPLC measurement of formed colistin in pharmacokinetic studies using the same products. This may provide new information on the metabolic properties of the prodrug. Other studies using HPLC in tandem with this FTIR method can also prove to be powerful strategies for elucidating the chemistry of this complicated prodrug (36, 37). For instance, HPLC separation of methanesulfonated colistin A and B products using a method such as that described by Bai et al. (10) followed by FTIR analysis can indicate whether one component is more heavily methanesulfonated. Time course studies may determine whether the methanesulfonate is a more labile leaving group on one component or the other. Since the prodrug lacks antimicrobial activity (27), these studies may provide information on the relative efficacy of the components and suggest ways to improve the use of this important drug.

Another important limitation of FTIR is that single bands are specific for chemical moieties within larger molecules rather than for the molecule as a whole. This is another reason to pursue whole-spectrum analysis methods like partial least-squares analysis for this application. As shown in this paper, plasma proteins can be effectively separated from the CMS sample. However, other drugs in the bloodstream that contain C-N or S-O bonds may coelute from SPE with CMS and interfere with quantification by the method reported here. The same type of interference may occur in a method developed using urine or other biological fluids. Vigilance is therefore required to ensure interfering compounds are separated from CMS by SPE or other methods prior to FTIR evaluation. Whole-spectrum analysis can be a powerful strategy for direct, specific detection of CMS. As discussed in the previous paragraph, the use of HPLC separation in combination with FTIR detection can also be used to ensure specificity (36, 37).

In conclusion, we report here a novel approach to detecting and quantifying colistin methanesulfonate (CMS) using ATR FTIR. By detecting molecular vibrations associated with the methanesulfonate groups, FTIR offers a method of direct CMS detection that does not require prior conversion to colistin and in which formed colistin does not interfere. To our knowledge, this is the first such assay. This method can be used to determine CMS concentration in pharmaceutical formulations, which can be translated into equivalent potential colistin units or used directly to remove ambiguity in dosage labeling (7). The method can measure as little as 50 μg/ml of CMS derived from aqueous solution and, given various methods of reporting CMS equivalencies in the literature and on medication packaging, may be a useful tool for directly determining the methanesulfonation of CMS in these products. ATR FTIR can also be used to follow the time course of CMS conversion to colistin and to detect CMS extracted from human plasma. With further development, this assay may be a powerful tool for adding to our knowledge base on this important antibiotic prodrug.

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